

Citrullinated Vimentin Presented on MHC-II in Tumor Cells Is a Target for CD4⁺ T-Cell-Mediated Antitumor Immunity

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Abstract

Stressful conditions in the harsh tumor microenvironment induce autophagy in cancer cells as a mechanism to promote their survival. However, autophagy also causes post-translational modification of proteins that are recognized by the immune system. In particular, modified self-antigens can trigger CD4⁺ T-cell responses that might be exploited to boost antitumor immune defenses. In this study, we investigated the ability of CD4 cells to target tumor-specific self-antigens modified by citrullination, which converts arginine residues in proteins to citrulline. Focusing on the intermediate filament protein vimentin, which is frequently citrullinated in cells during epithelial-to-mesenchymal transition of metastasizing epithelial tumors, we

generated citrullinated vimentin peptides for immunization experiments in mice. Immunization with these peptides induced IFN γ - and granzyme B-secreting CD4 T cells in response to autophagic tumor targets. Remarkably, a single immunization with modified peptide, up to 14 days after tumor implant, resulted in long-term survival in 60% to 90% of animals with no associated toxicity. This antitumor response was dependent on CD4 cells and not CD8⁺ T cells. These results show how CD4 cells can mediate potent antitumor responses against modified self-epitopes presented on tumor cells, and they illustrate for the first time how the citrullinated peptides may offer especially attractive vaccine targets for cancer therapy. *Cancer Res*; 76(3); 548–60. ©2015 AACR.

Introduction

One of the limitations of tumor immunotherapy is the immunosuppressive tumor environment. This can be partly abrogated by removing regulatory T cells (Tregs), inhibiting myeloid suppressor cells, neutralizing PD-L1 (1), or enhancing T-cell responsiveness using checkpoint inhibitors targeting CTLA-4 and PD-1 (2–5). The checkpoint inhibitors have had a dramatic effect on survival in melanoma patients but are associated with 10% to 53% grade 3/4 autoimmune complications (2, 3, 5). CD4 T cells are the orchestrators of the immune response and, when activated within a tumour, release IFNs that act directly to upregulate MHC and stimulate the release of proinflammatory chemokines to promote further immune infiltration (6–8). The CD4 repertoire to self-antigens is highly tolerized (9–11). In contrast, CD4 T cells recognizing modified self-epitopes have been shown to play a role in the pathophysiology of several autoimmune diseases such as

rheumatoid arthritis (RA), collagen II-induced arthritis, sarcoidosis, celiac disease, and psoriasis (12–15). One of these common modifications is citrullination of arginine, which involves the conversion of the positively charged aldimine group (=NH) of arginine to the neutrally charged ketone group (=O) of citrulline. Citrullination is mediated by peptidylarginine deiminases (PAD), which are a family of calcium-dependent enzymes found in a variety of tissues. A recent elegant report by Ireland and colleagues demonstrates that the presentation of citrullinated T-cell epitopes on APCs is also dependent upon autophagy and PAD activity (16). This process has also been demonstrated to be an efficient mechanism to enable processing of endogenous antigens for presentation on MHC class II molecules in professional APCs as well as epithelial cells (17, 18). It has also been demonstrated that induction of autophagy increases the presentation of cytosolic proteins in B cells and thymic epithelial cells (19, 20). Autophagy is constitutive in APCs but is only induced by stress in other cells (21). Thus T cells recognizing citrullinated epitopes have no target on normal healthy cells. Autophagy is triggered by stress such as hypoxia and nutrient starvation and is upregulated to promote tumor survival (21). One of the most frequently citrullinated proteins is vimentin, a cytoskeletal protein expressed by all mesenchymal cells and one of the first proteins to be upregulated during epithelial to mesenchymal transition of metastasizing epithelial tumors. In addition, it has also recently been shown to be citrullinated in some human tumor cell lines (22).

In this study, we show that it is possible to induce CD4 responses to citrullinated self-peptide epitopes with minimal reactivity to the unmodified sequence. We are the first to demonstrate that tumor cells present citrullinated epitopes. T cells specific for these citrullinated peptide epitopes can target tumor

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cells to elicit strong antitumor effects *in vivo*, thus providing the first evidence for the use of citrullinated peptides as vaccine targets in cancer therapy.

Materials and Methods

Cell lines and culture

The T-cell/B-cell hybrid cell line T2 stably transfected with functional class II DR4 (DRB1*0401; T2 DR4) has been described (23). Murine pancreatic cancer cell line Pan02 was obtained from the DCTD Tumor/Cell line repository at National Cancer Institute. The murine melanoma B16F1 cell line and murine lung carcinoma line LLC/2 were obtained from the ATCC. All cell lines were cultured in RPMI medium 1640 (GIBCO/BRL) supplemented with 10% FCS, L-glutamine (2 mmol/L), and sodium bicarbonate buffered. Cell lines utilized are mycoplasma free, authenticated by suppliers (STR profiling), and used within ten passages.

To stress tumor target cells for *in vitro* assays, cells were treated with 0.1 mol/L citric acid (pH3.0) containing 1% BSA at 4°C for 2 minutes, washed with media, and cultured in absence of serum for 20 hours at 37°C. Autophagy and PAD inhibitors, 3-methyladenine (Sigma) and CI-amidine (Calbiochem), at final concentrations of 10 mmol/L and 50 µg/mL, respectively, or rapamycin (Calbiochem) and bafilomycin (Calbiochem) at final concentrations of 800 nmol and 500 nmol, respectively, were added for the 20-hour culture in serum-free media.

Peptides

Peptides aa14-32cit (MFGGSGTSS-cit-PSSN-cit-SYVT), aa26-44cit (SN-cit-SYVTTST-cit-TYSLGSAL), aa28-49wt (RSYVTTSTR-TYSLGSALRPSTS), aa28-49cit (cit-SYVTTST-cit-TYSLGSAL-cit-PSTS), aa31-50cit (VITST-cit-TYSLGSAL-cit-PSTS-cit), aa36-54cit (cit-TYSLGSAL-cit-PSTS-cit-SLYS), aa46-64cit (PSTS-cit-SLYSSS-PGGAYVT-cit-S), aa401-419cit (cit-KLEGEES-cit-ISLPLPTFS), aa406-425cit (GEES-cit-ISLPLPTFSSLNL-cit-E), aa415-433wt (LPNFSSLNLRNLDLPL), and aa415-433cit (LPNFSSLNL-cit-ETNLDLPL) were synthesized at >90% purity by GenScript and stored lyophilized at -80°C. On the day of use they were reconstituted to the appropriate concentration in 10% dimethyl formamide.

Plasmids

To construct the plasmid pVITRO 2 Chimeric HLA-DR401, cDNA was generated from mRNA isolated from the splenocytes of transgenic HLA-DR4 mice. This was used to amplify the chimeric α and β chains separately using forward and reverse primers that incorporated a FspI/EcoRI and BamHI/SalI sites, respectively. Following sequence confirmation full-length chimeric α chain comprising murine H2-Ea with the human HLA-DRA α 1 domain was ligated into the FspI/EcoRI mcs2 of the vector pVITRO2-hygro-mcs (Invivogen). The β chain comprising murine H2-Eb with human DRB1*0401 Beta 1 domain was then inserted into the BamHI/SalI mcs1 of the vector alongside the chimeric α chain.

To generate the IFN γ -inducible plasmid pDCGAS chimeric HLA-DR401, the chimeric α and β chains, were cloned into the pDCOrig vector described elsewhere (24) in replacement of the heavy and light chain. The IFN γ -inducible promoter consisting of a TATA box and the GAS (IFN γ activated sequence) direct repeat enhancer element was amplified by PCR utilizing the vector pGAS-Luc (Agilent) as a template. The CMV promoter within each cassette was excised and replaced with the IFN γ -inducible

promoter driving expression of the HLA-DR401 chains within the pDCOrig vector backbone.

The plasmid MC207329 (Origene) encoding untagged full-length murine Myelin Oligodendrocyte Glycoprotein cDNA (NM_010814) was cloned into the SgfI/MluI mcs of the mammalian expression vector pCMV 6.

After sequence confirmation endotoxin-free plasmid DNA was generated using the Endofree Maxiprep Kit (Qiagen).

Transfection and flow cytometry

B16F1 melanoma, LLC/2 lung carcinoma, and Pan02 pancreatic cancer cells were transfected using the Lipofectamine Transfection Reagent (Invitrogen) with 4 µg of the plasmid pVITRO 2 Chimeric HLA-DR401 that encodes both full-length chimeric α and β chains according to the manufacturer's instructions. The B16F1 cell line was transfected with either the MOG plasmid or the pDC GAS chimeric HLA-DR401 or the pVITRO 2 chimeric HLA-DR401 plasmids where chimeric HLA-DR401 is under expression of the IFN γ -inducible promoter or the constitutive promoter that drive high-level expression respectively.

Transfected cells were selected by growth in the presence of G418 (500 µg/mL), zeocin (300 µg/mL), or hygromycin B (300 µg/mL). Lines were cloned by limiting dilution and expression was confirmed by flow cytometry using the HLA-DR PE conjugated antibody (clone L243) from eBioscience or the murine MOG antibody (clone 8-18C5, Millipore) with the anti-mouse IgG1 AlexaFluor 647 secondary antibody (Life Technologies). Cells transfected with the IFN γ -inducible plasmid where incubated overnight in the absence or presence of murine IFN γ (30 ng/mL, Gibco Life Technologies) before staining with the antibody.

HLADR*0401-binding studies

In brief, peptides of interest were mixed with a predetermined concentration of biotinylated influenza hemagglutinin HA₃₀₆₋₃₁₈ reference peptide at increasing concentrations and added to plate bound HLA-DR*0401. The amount of biotinylated reference peptide binding to HLA DR*0401 was quantified using a streptavidin-linked enzyme step followed by detection with chromogenic substrate. Maximal binding is taken as the value achieved by biotinylated HA₃₀₆₋₃₁₈ peptide alone. As a positive control unlabeled HA₃₀₆₋₃₁₈ peptide was used to compete with the biotinylated version.

Immunization protocol

HLA-DR4 mice (Model # 4149, Taconic) or C57Bl/6J mice (Charles River) aged between 8 weeks and 12 weeks were used. All work was carried out at Nottingham Trent University (Nottingham, United Kingdom) under a Home Office approved project license. For all studies, the mice were randomized into different groups and not blinded to the investigators. Peptides were dissolved in 10% dimethylformamide to 1 mg/mL and then emulsified with CpG and MPLA 6 µg/mouse of each (Invivogen). Peptides (25 µg/mouse) were injected subcutaneously at the base of the tail. Mice were immunized on day 0 and spleens were removed for analysis at day 12. Antibodies to IFN γ (clone XMG1.2), IL17 (clone 17F3), CD8 (clone 2.43), and CD4 (clone GK1.5) were purchased from BioXcell.

For tumor challenge experiments, mice were challenged with 2.5×10^4 B16 DR4 cells, 1×10^5 B16 MOG cells or 1.5×10^6 LLC/2 DR4 cells subcutaneously on the right flank 3 days before primary immunization (unless stated otherwise) and then were

immunized as above. The antibodies to IFN γ (300 μ g/dose) and IL17 (200 μ g/dose) were administered via the intraperitoneal (i.p.) route in saline at days 2, 7, 10, and 14 after tumor implant. The CD4-specific antibody (500 μ g/dose) was administered i.p. in saline at days 2 and 8 after tumor implant. Tumor growth was monitored at 3 to 4 days intervals and mice were humanely euthanized once tumor reached ≥ 10 mm in diameter.

Ex vivo Elispot assay

Elispot assays were performed using murine IFN γ or IL17 capture and detection reagents according to the manufacturer's instructions (Mabtech). In brief, the IFN γ or IL17-specific antibodies were coated onto wells of 96-well Immobilon-P plate. Synthetic peptides (10 μ g/mL) and 5×10^5 per well splenocytes were added to the wells of the plate in triplicate. Tumor target cells were added where relevant at 5×10^4 /well in triplicate and plates incubated for 40 hours at 37°C. After incubation, captured IFN γ or IL17 were detected by biotinylated specific IFN γ or IL-17 antibodies and developed with a streptavidin alkaline phosphatase and chromogenic substrate. Spots were analyzed and counted using an automated plate reader (Cellular Technologies Ltd).

Ex vivo depletion of CD4 or CD8 cells from splenocyte cultures

Splenocytes were subject to positive isolation of CD4 or CD8 cells using antibody-coated magnetic beads (Miltenyi Biotec) according to manufacturer's instructions. For MHC blocking studies 20 μ g/mL of the HLA-DR (clone L243)-specific antibody was added to Elispot assays.

Granzyme B Elisa

Supernatant from *ex vivo* IFN γ Elispot assays on splenocytes was removed after 40 hours and assessed for Granzyme B by Elisa assay (R&D Systems) according to manufacturer's instructions.

Western blotting

Cell lines were cultured under different conditions as detailed above with the inclusion of E64d and pepstatin A protease inhibitors (10 μ g/mL, Calbiochem). Cell lysates were prepared in RIPA buffer containing protease inhibitor cocktail (Sigma) and proteins separated on a 12% NuPage Bis-Tris Gel (Invitrogen) followed by transfer onto PVDF membrane. The membrane was probed with antibodies to human/mouse LC3A/B (clone D3U4C, Cell Signaling Technology) and β -actin (clone AC-15, Sigma). Proteins were visualized using fluorescent secondary antibodies against mouse (for LC3A/B) or rabbit (for β -actin) and a Licor detection system. Quantification was performed using Image Studio software and the intensity of respective bands was normalized to β -actin loading control.

p62 Elisa

Cell lines were cultured under different conditions as detailed above. Lysates were prepared in RIPA buffer with PMSF, DNase, and protease inhibitor cocktail (Calbiochem) and analyzed by p62 Elisa according to manufacturer's instructions (Enzo Life Sciences).

Proliferation assays on human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from normal donors and melanoma patients (ranging from early to advanced disease classification) and subject to CFSE labeling

(where specified). Cells were cultured with 10 μ g/mL peptide and analyzed at day 11 dilution of CFSE by flow cytometry combined with staining for CD4 PE-Cy5 (clone RPA-T4, eBioscience) and CD8 efluor 450 (clone RPA-T8, eBioscience) or for 3 H thymidine uptake.

Statistical analysis

Comparative analysis of the Elispot results was performed by applying unpaired Student *t* test with values of *P* calculated accordingly. Comparison of tumor survival was assessed by log-rank test using the GraphPad Prism software. *P* < 0.05 values were considered statistically significant and *P* < 0.05 values were considered highly significant. The error bars shown in the figures represent the mean + SD.

Results

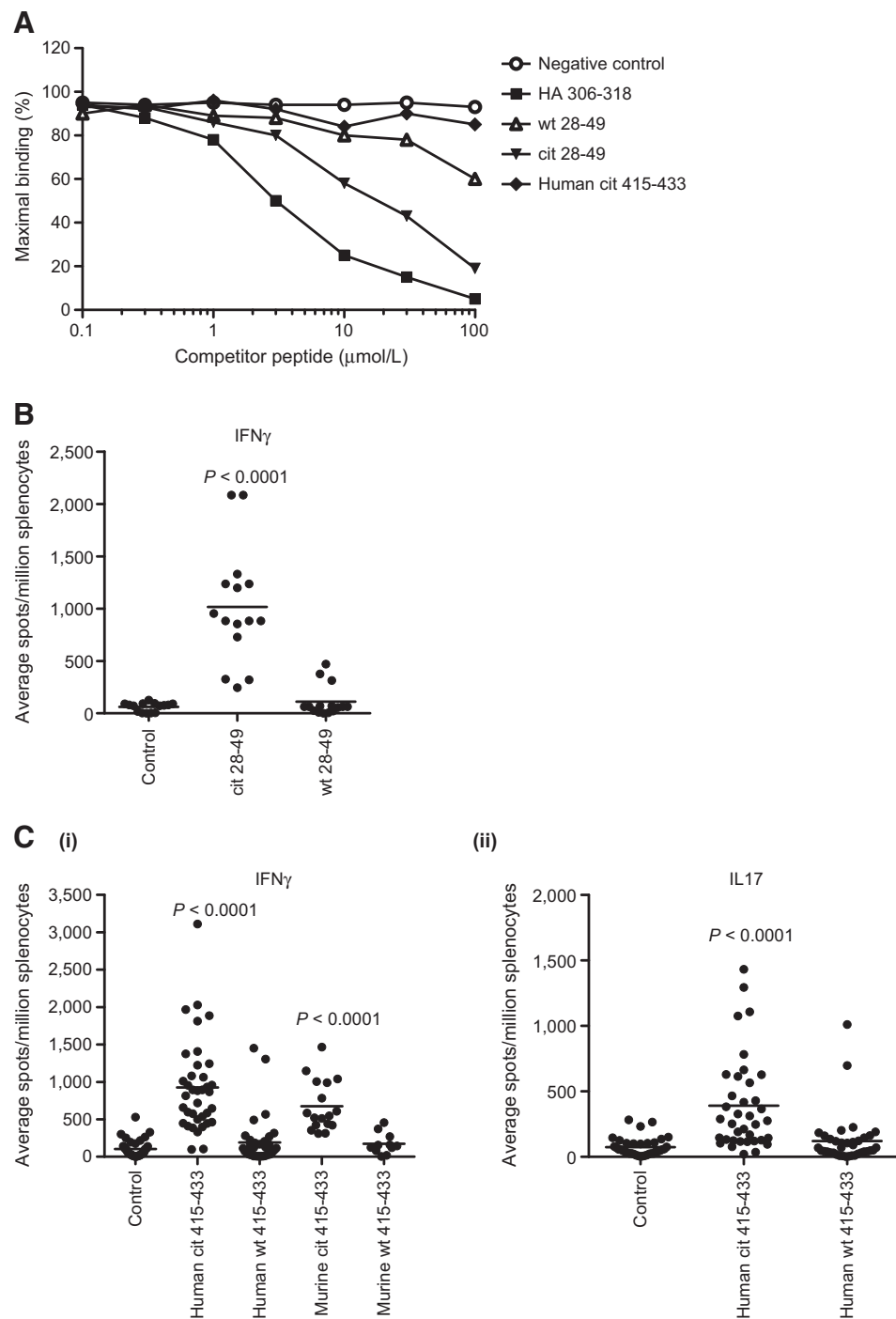
Peptide vaccination induces high-frequency CD4 responses to two citrullinated vimentin epitopes in HLA-DR4 transgenic mice

Two citrullinated peptide epitopes from vimentin (aa28-49 and aa415-433) were selected for this study as they had been previously shown to induce T-cell responses in mice and humans (25). The vimentin aa 26-44 peptide from this published study was changed to encompass aa 28-49, which allowed inclusion of a third citrulline residue and was shown to induce a stronger immune response compared with the same peptides containing only two citrulline residues (Supplementary Fig. S1). This aa28-49 peptide is completely homologous in mice and humans. To confirm HLA-DR4 binding, the wild-type and citrullinated aa28-49 and human aa415-433 peptides were screened in HLA-DR4-binding competition assays with influenza hemagglutinin HA306-318 peptide. Vimentin aa28-49 citrullinated peptide shows stronger binding to HLA-DR*0401 compared with the wild-type version, whereas citrullinated aa415-433 peptide showed weaker binding (Fig. 1A).

HLA-DR4 transgenic mice were immunized with citrullinated aa28-49 or aa415-433 peptides in combination with CpG and MPLA adjuvants. Citrullinated aa28-49 and aa415-433 peptides stimulated strong IFN γ responses against the citrullinated peptides and no significant responses to the wild-type peptide compared with control (Fig. 1B and C, i). Citrullinated aa415-433 also stimulated strong IL17 responses against the citrullinated peptide and no significant responses to the wild-type peptide (Fig. 1C, ii). No significant IL10 responses were observed specific for either the aa415-433 or aa28-49 peptides (data not shown). Since the aa415-433 epitope has two amino acid differences between human and murine sequences, cross reactivity to the murine sequence was tested. Responses induced with the human sequence cross-reacted with the murine peptide with no wild-type reactivity (Fig. 1C, i). Mice also made similar responses if immunized with the murine aa415-433 peptide (Supplementary Fig. S2).

Citrullinated epitope-specific responses are CD4 mediated and possess cytotoxic capability

Responses specific for citrullinated aa415-433 and aa28-49 peptides were shown to be CD4 mediated by depletion of CD4 cells before analysis or addition of MHC class II blocking antibody (Fig. 2A and B). Depletion of CD8 cells had no effect upon the peptide-specific responses (Supplementary Fig. S3). Absence of CD8 epitopes within the sequences was confirmed by

**Figure 1.**

Citrullinated peptides induce IFN γ and IL17 responses. A, HLA-binding affinity of citrullinated (cit) and wild-type (wt) vimentin peptides. B and C, HLA-DR4 transgenic mice were immunized with citrullinated vimentin aa28-49 (B) or aa415-433 (C) peptides and splenocytes analyzed for peptide-specific IFN γ or IL17 responses to the citrullinated or wild-type peptides in Elispot assay. All studies are representative of at least three independent experiments in which $n = 3$. Mean shown as line.

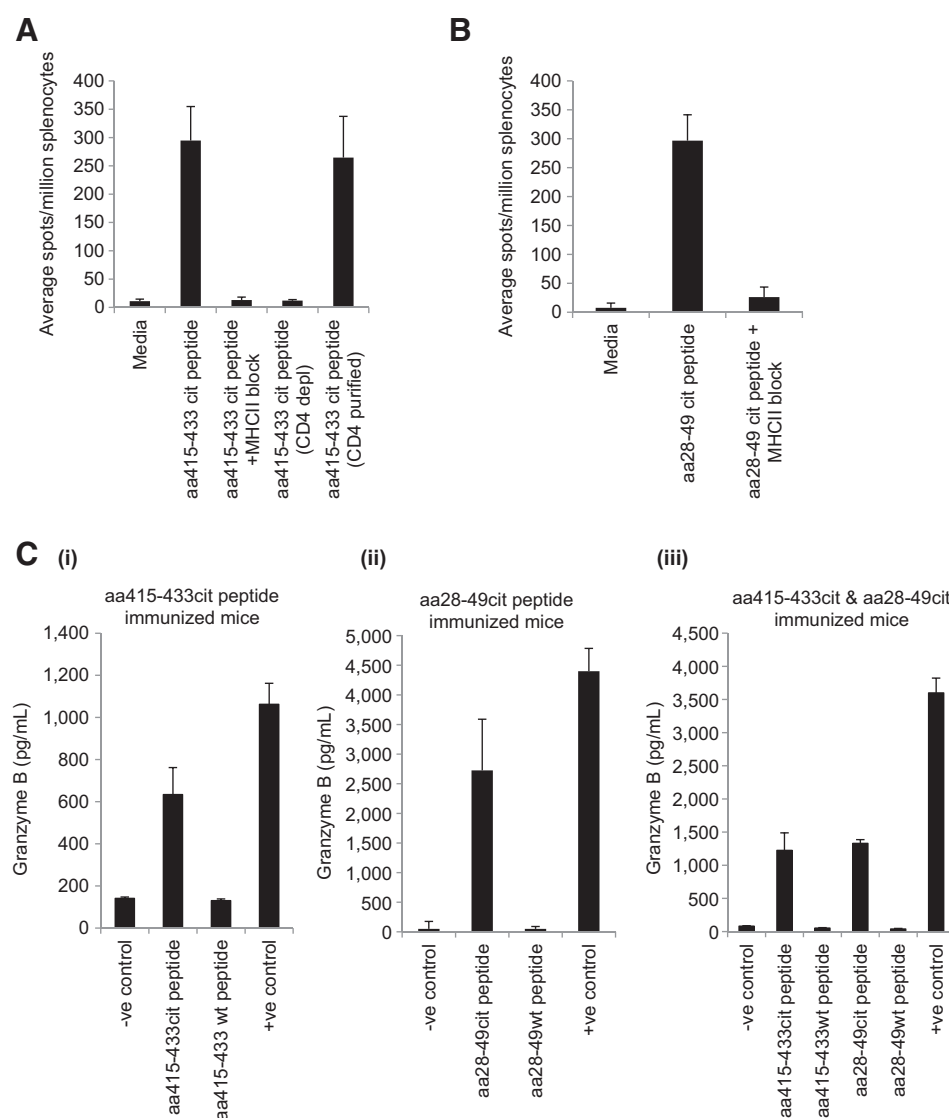
immunization of C57Bl/6 and HHDII/DR1 mice. They failed to raise a response, suggesting that the epitopes are not presented on H-2Kb, H-2Db, I-Ab, HLA-A2, or HLA-DR*0101 (data not shown).

To assess whether citrullinated peptide-specific CD4 responses were capable of cytotoxicity as well as cytokine release, the secretion of granzyme B was analyzed. Splenocytes from mice immunized with either citrullinated aa28-49 or aa415-433 peptides or both demonstrate release of granzyme B, a marker of

cytotoxicity, upon stimulation with aa415-433 ($P < 0.0001$) and aa28-49 ($P < 0.0001$) citrullinated peptides in contrast with the wild-type versions (Fig. 2C).

Tumor cells present citrullinated epitopes via autophagy and PAD-dependent mechanisms

The ability of the citrullinated epitope-specific responses to recognize tumors was assessed. T cells stimulated with the combination of peptides failed to recognize targets that expressed

**Figure 2.**

Citrullinated vimentin-specific responses are CD4 mediated and produce granzyme B. A and B, splenocytes from mice immunized with citrullinated aa415-433 (A) or aa28-49 (B) peptides were subject to MHC class II blocking or CD4 depletion and peptide-specific IFN γ responses assessed in Elispot assay. C, splenocytes from citrullinated peptide immunized mice were tested for peptide-specific granzyme B release by Elisa. All studies are representative of at least three independent experiments in which $n = 3$. Data are presented as mean + SD.

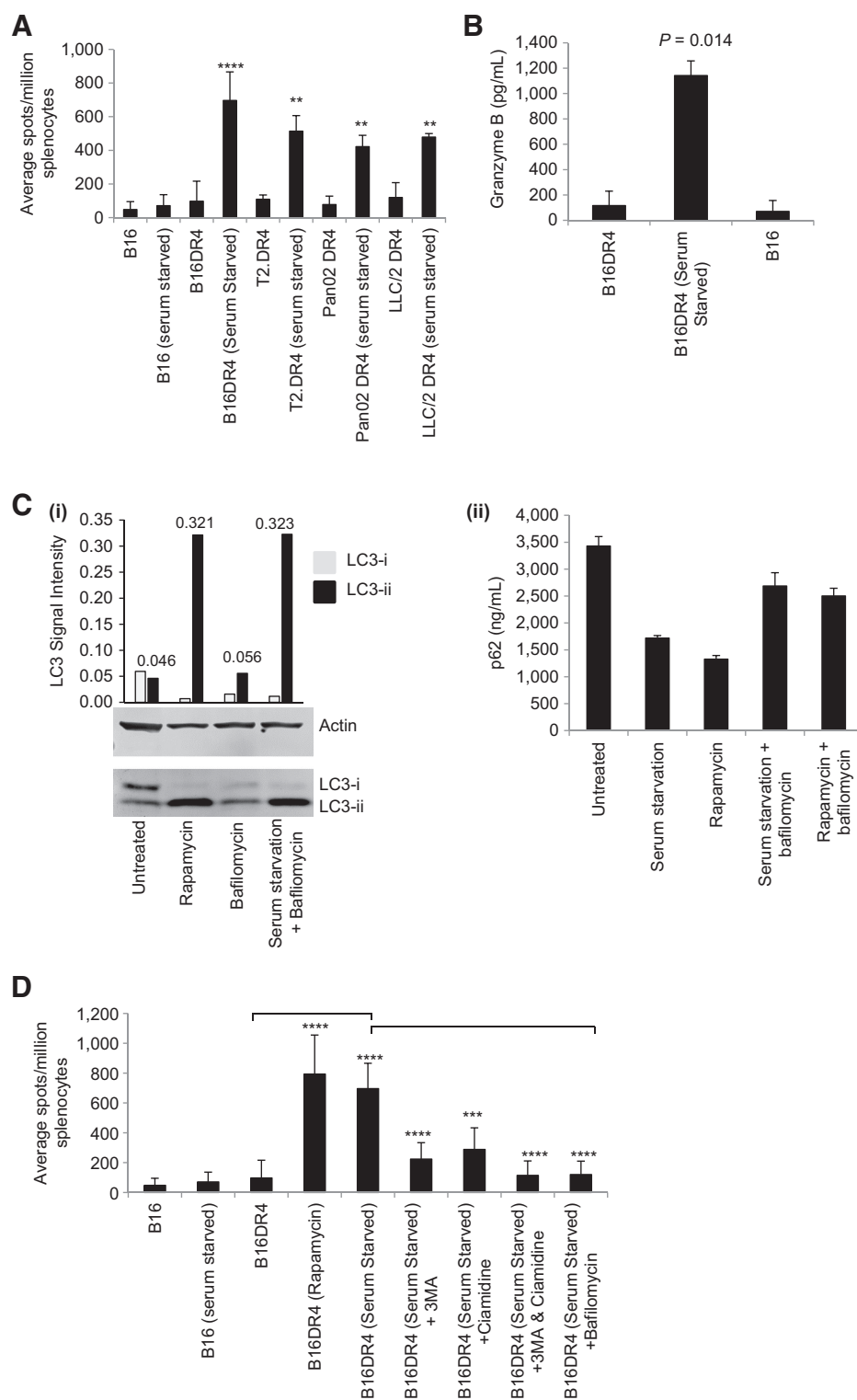
HLA-DR4 under normal conditions. However, if the cells were stressed by serum starvation good recognition of the mouse cell lines and the human T2 DR4 cell line was seen, suggesting that all these cell lines present the citrullinated epitope when stressed (Fig. 3A). Granzyme B is also released upon response to serum-starved B16DR4 tumor target cells, suggesting CD4 killing of tumor targets presenting the citrullinated epitopes (Fig. 3B).

Since it has been suggested that proteins can undergo citrullination via PAD activity in autophagic vesicles, we considered whether autophagy was being induced in the cell lines as a result of the stress imposed by serum starvation. The T-cell recognition assay was repeated and induction of autophagy assessed in the presence of inducers or inhibitors of autophagy and inhibitors of PAD. Immunoblot analysis of B16DR4 lysates from cells treated with the autophagy inducer rapamycin results in higher levels of expression of the autophagy marker LC3ii, indicating an increase in autophagic vesicles (Fig. 3C, i). The inclusion of bafilomycin, which prevents autophagosome fusion with lysosomes, thus inhibiting protein degradation, in the presence of serum starvation increased LC3ii expression compared with untreated cells,

indicating accumulation of autophagosomes in the serum-starved cells (Fig. 3C, i). The polyubiquitin-binding protein p62/SQSTM1 is known to bind to LC3, it is degraded by autophagy and is commonly used alongside LC3 as an indicator of autophagy. The induction of autophagy by serum starvation or rapamycin treatment is observed with reduction in levels of p62, which is increased on addition of bafilomycin (Fig. 3C, ii). T-cell recognition of tumor target cells was increased in the presence of rapamycin and blocked on serum starvation in the presence of bafilomycin and the autophagy inhibitor 3-methyladenine (3-MA). T-cell recognition of serum-starved tumor cells was also decreased in the presence of PAD inhibitor CI-amidine, indicating that the presentation and T-cell recognition of citrullinated peptides are both autophagy and PAD dependent (Fig. 3D).

Citrullinated vimentin-specific CD4 responses show antitumor activity *in vivo* against established tumors

To assess the antitumor activity *in vivo*, HLA-DR4 transgenic mice were implanted with B16F1 tumors expressing HLA-DR4 followed by immunization with single or combination of

**Figure 3.**

Citrullinated peptides are presented on tumor cells in a PAD and autophagy-dependent manner. A and B, splenocytes from mice immunized with both citrullinated peptides were assessed for the ability to recognize tumor cells in IFN γ Elispot assay (A) or by granzyme B Elisa (B). Values are relative to tumor cell lines grown in complete medium. **, $P < 0.01$; ****, $P < 0.0001$. Representative of at least three independent experiments where $n = 3$. C, Western blot analysis of B16DR4 tumor cell lysates probed for the LC3 autophagy marker, with associated histogram summarizing the densitometric analysis of the LC3-I and LC3-II bands normalized to β -actin control (i) and p62 Elisa showing induction of autophagy when treated with rapamycin, bafilomycin, and serum starvation (ii). Values were averaged with data from two independent experiments in which $n = 3$ and mean \pm SD. D, recognition of nutrient-starved tumor cells and inhibition in the presence of autophagy and PAD inhibitors. Representative of at least three independent experiments in which $n = 3$. Values are relative to B16DR4 cells grown in the presence or absence of serum as indicated. ***, $P < 0.001$; ****, $P < 0.0001$. Data are presented as mean \pm SD.

citrullinated peptides. Mice immunized with citrullinated peptides demonstrate strong antitumor responses (Fig. 4A).

To test whether the immune responses were as efficient against more established tumors, vaccination was delayed until 7, 10, or

14 days after B16DR4 tumor implant. A single vaccination with both peptides shows enhanced survival in mice even when vaccination is delayed until day 14 after tumor implant (Fig. 4B and C) or if tumor is initiated with 6-fold higher tumor load (Fig. 4D).

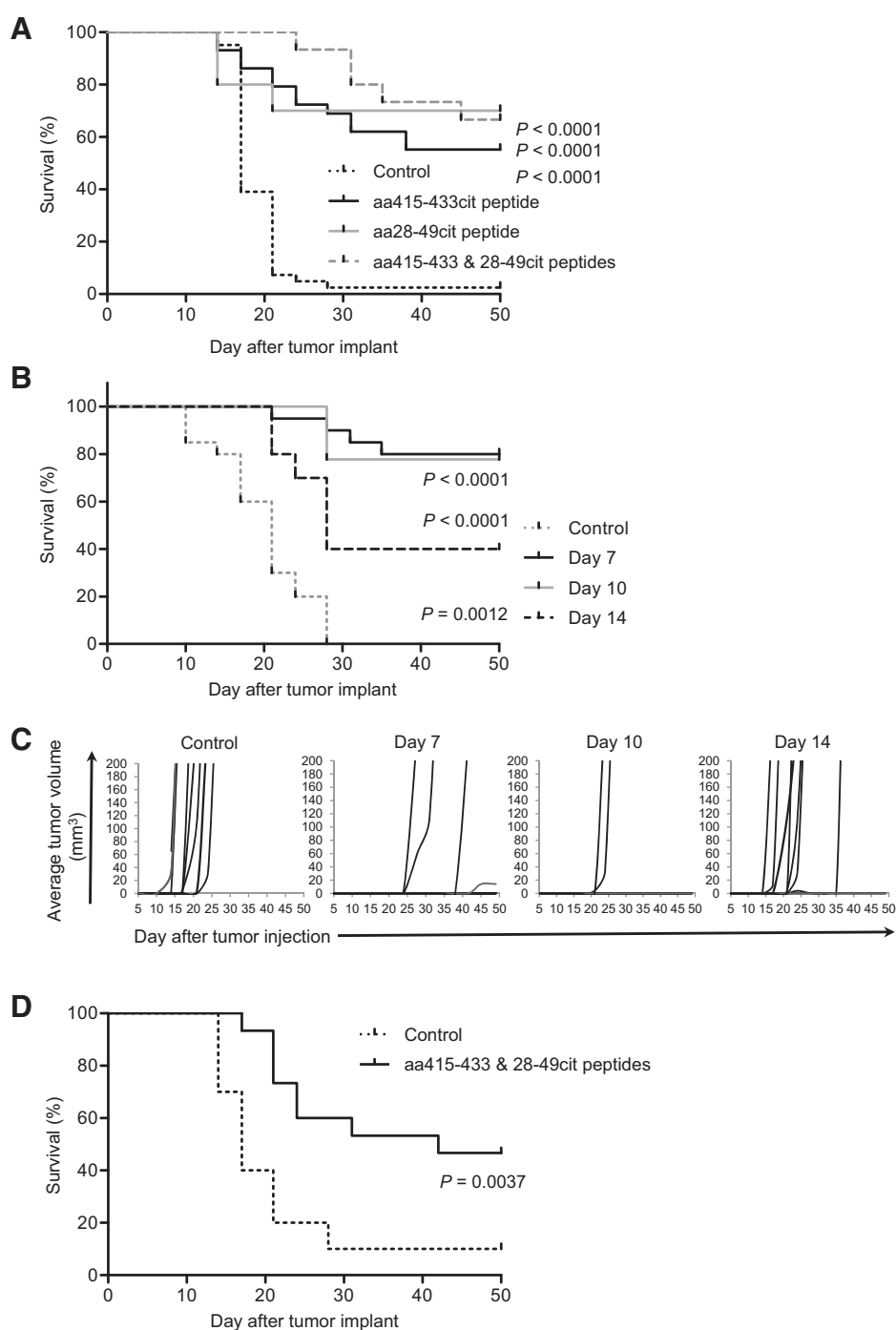


Figure 4. Citrullinated vimentin peptide vaccination induces efficient tumor therapy. A, survival of mice challenged with B16DR4 tumor and immunized 4 days after tumor implant with citrullinated vimentin aa28-49, aa415-433 peptide, or the combination. B and C, survival (B) and tumor growth (C) curves of mice challenged with B16DR4 tumor and immunized 7, 10, or 14 days after tumor implant with citrullinated vimentin aa28-49 and aa415-433 peptides. Studies are representative of at least two independent experiments in which $n = 9$. D, survival of mice challenged with high tumor load of B16DR4 cells (1.5×10^5) and immunized 4 days post-tumor implant in combination with citrullinated vimentin aa28-49 and aa415-433 peptides. Study is representative of data from at least two independent studies in which $n = 10$.

Antitumor responses induced by each peptide are CD4 mediated and IFN γ dependent

To determine whether these antitumor responses were mediated by CD4 T cells, mice were treated with anti-CD4 antibody *in vivo*. Vaccination combined with CD4 T-cell depletion totally abrogates the antitumor response mediated by both peptides (Fig. 5A). The influence of CD8 cells on the antitumor effect was assessed by use of anti-CD8 antibody

combined with vaccination. Depletion of CD8 cells had no significant effect upon the antitumor effect (Fig. 5B). Both citrullinated peptides induce high-frequency IFN γ responses. Blockade of IFN γ *in vivo* inhibits both citrullinated peptide-specific antitumor responses (Fig. 5C). Citrullinated aa415-433 also induces IL17 responses. Blockade of these *in vivo* had a weak, but significant, influence upon *in vivo* antitumor effects (Fig. 5D).

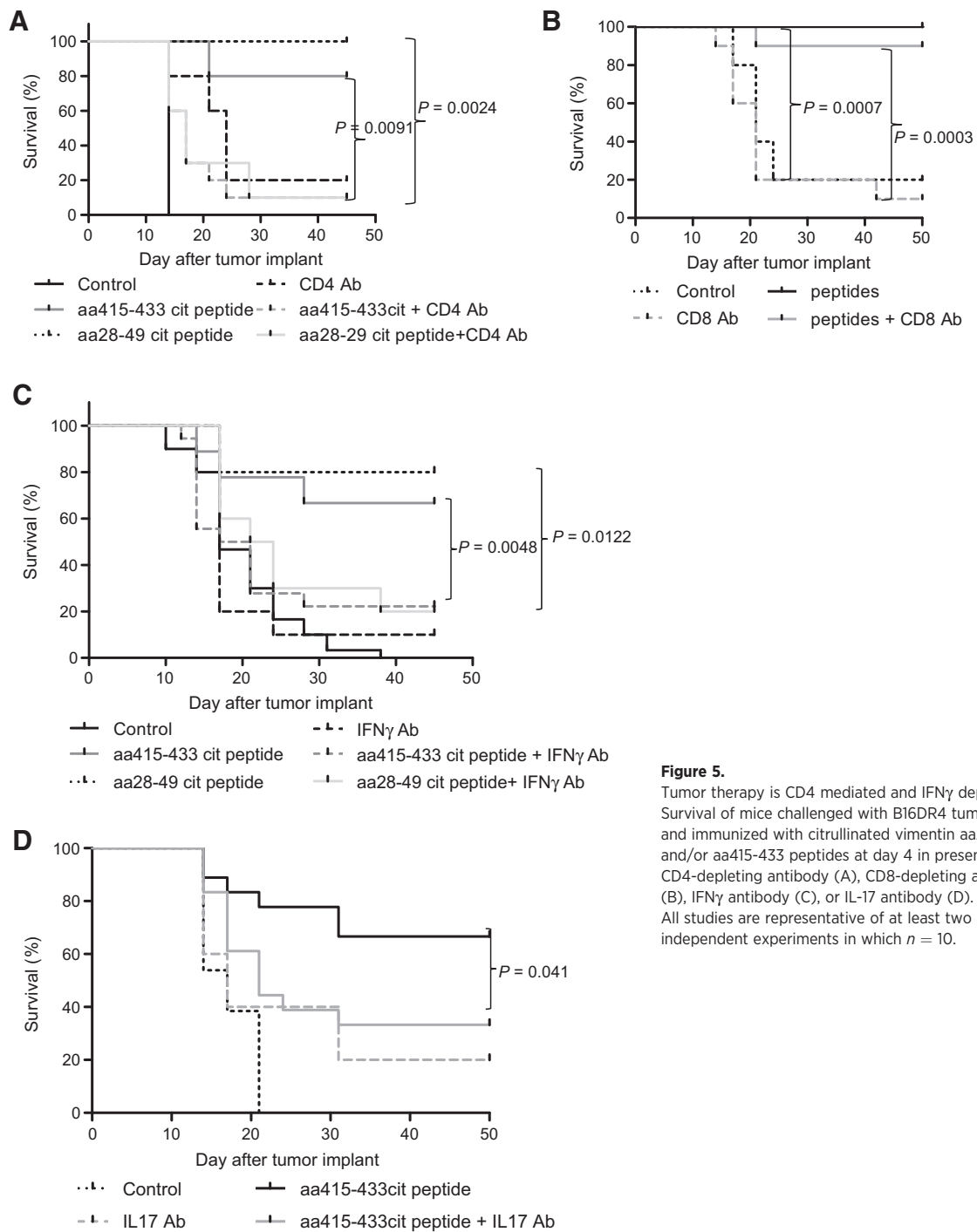


Figure 5. Tumor therapy is CD4 mediated and IFN γ dependent. Survival of mice challenged with B16DR4 tumor and immunized with citrullinated vimentin aa28-49 and/or aa415-433 peptides at day 4 in presence of CD4-depleting antibody (A), CD8-depleting antibody (B), IFN γ antibody (C), or IL-17 antibody (D). All studies are representative of at least two independent experiments in which $n = 10$.

Do the antitumor responses require HLA-DR4 expression by the tumor?

CD4 responses within tumor sites can be stimulated by APCs presenting tumor antigens. This can trigger the release of cytokines and chemokines promoting inflammation and recruitment of other effector cells. Alternatively, CD4 T cells can differentiate to cytotoxic cells expressing granzyme and Fas Ligand and directly

kill tumors expressing MHC-II. As the antitumor responses of the citrullinated peptides were not dependent upon CD8 T cells, this suggested that direct recognition of tumors by the CD4 T cells was important. To address this issue, HLA-DR4 transgenic mice were implanted with B16F1 tumors that do not express HLA-DR4 followed by immunization with the combination of citrullinated peptides. Mice immunized with citrullinated peptides showed a

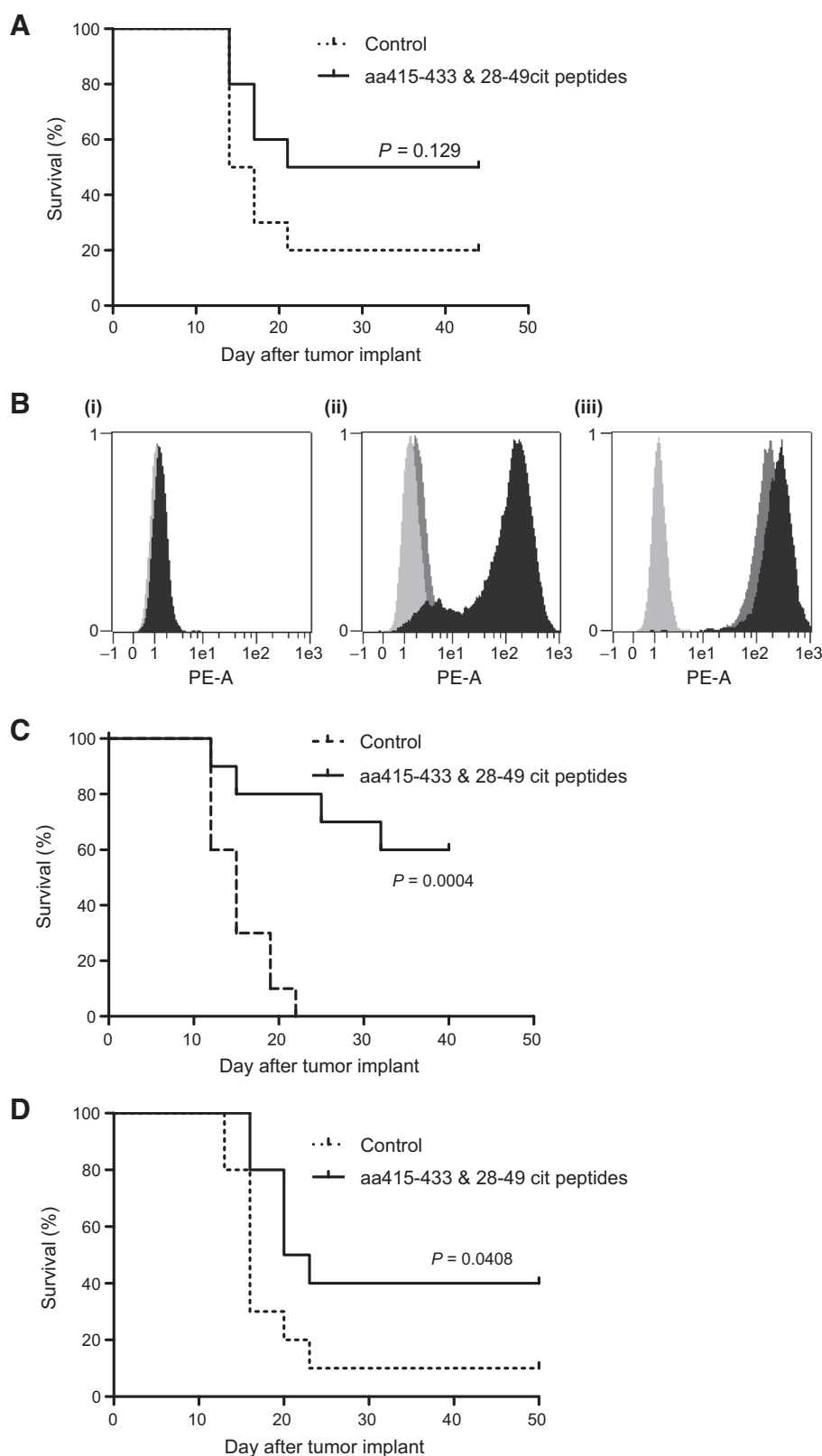


Figure 6.

Antitumor responses require expression of HLA-DR4 on the tumor. A, survival of mice challenged with B16F1 tumor cells knocked out for MHC class II were immunized 4 days after tumor implant in combination with citrullinated vimentin aa28-49 and aa415-433 peptides in which $n = 10$. B, normalized FACS profiles demonstrating expression of HLA-DR401 on the surface of B16F1 cells knocked out for murine MHC class II (i) transfected with the pDCGas HLA-DR401 IFN γ -inducible plasmid (ii) and with the constitutive high-level expression pViro 2 HLA-DR401 plasmid (iii) on incubation without (dark gray) and with (black) IFN γ . Light gray profiles represent unstained samples. C, survival of mice challenged with the transfected B16F1 IFN γ -inducible HLA-DR401 tumor cells and immunized 4 days after tumor implant in combination with citrullinated vimentin aa28-49 and aa415-433 peptides ($n = 10$). D, survival of mice challenged with LLC/2 HLA-DR401 tumor and immunized 4 days after tumor implant with both citrullinated vimentin aa28-49 and aa415-433 peptides ($n = 10$).

weak antitumor, which failed to show significance (Fig. 6A), suggesting direct recognition of HLA-DR4 on tumor cells was important for the antitumor response. Although, some melano-

mas constitutively express MHC-II, most only express MHCII following IFN γ stimulation. B16F1 were therefore transfected with HLA-DR4 under control of the mouse IFN γ -inducible

promoter GAS. IFN γ upregulation of HLA-DR4 was demonstrated on this tumor cell line (Fig. 6Bii). HLA-DR4 transgenic mice were implanted with IFN γ -inducible HLA-DR4 B16F1 tumors and immunized with the combination of citrullinated peptides. Mice immunized with citrullinated peptides show a strong antitumor response (Fig. 6C), suggesting that the CD4 T cells secreted sufficient IFN γ to induce HLA-DR4 expression, which was important for the antitumor response. To show that IFN γ alone was not sufficient to induce an antitumor response, the mouse Lewis lung carcinoma LLC/2, which is unresponsive to IFN γ , was transfected with HLA-DR4. Mice immunized with the combination of citrullinated peptides showed a significant antitumor response ($P = 0.0408$, Fig. 6D), again suggesting that direct recognition of HLA-DR4 on tumor cells was important for the antitumor response.

Does citrullinated vimentin represent a novel class of tumor associated antigen?

To demonstrate that citrullinated vimentin represents a tumor-associated antigen to which T cells have not been tolerized, it was necessary to show responses in wild-type mice and in human PBMCs *in vitro*. Myelin oligodendrocyte glycoprotein (MOG) aa35-55 is a known citrullinated epitope in C57Bl mice. It contains two arginines at positions 41 and 46 that when changed to citrulline induce immune responses (26). The epitope with citrulline at position 46 is known to show lower cross-reactivity with the wild-type sequence and thus minimize autoimmune effects. We immunized mice with this peptide and showed strong T-cell responses specific to the citrullinated peptide (Fig. 7A). MOG was transfected into our B16F1 tumor model and 4 days following tumor implant, mice were immunized with the MOG 35-55 (cit46) citrullinated peptide. Strong antitumor responses were induced in 70% of mice (Fig. 7B). These results demonstrate that tumors can citrullinate proteins in wild-type mice. To determine whether wild-type mice could also respond to citrullinated vimentin C57Bl mice were immunized with pools of citrullinated vimentin peptides encompassing all regions containing an arginine as published in Fietsma and colleagues but no responses were observed. Vimentin was further analyzed for I-Ab motifs that encompassed an arginine. Six citrullinated peptides were synthesized and used to screen C57Bl mice. Two of these showed strong citrulline-specific T-cell responses (Fig. 7C). Interestingly the strongest response is to sequence aa31-50, which cross-reacts with the peptide encoding sequence aa26-44. Both of these contain part of the aa28-49 sequence. It appears that it is the citrulline at position 36 that is important in this response (Supplementary Table S1). We have demonstrated in DR4 mice that this region is naturally citrullinated. Another response to the citrullinated peptide spanning aa401-419 was detected in C57Bl/6 mice. This response did not cross-react with aa406-425 sequence or aa415-433 sequence. These data demonstrate that responses to citrullinated epitopes have not been deleted in wild-type mice and confirm that our responses in the HLA-DR4 transgenic mice are not an artifact of the transgenic model. Furthermore, melanoma patients were screened with the citrullinated peptides aa415-433 and aa28-49. Eight of twenty-three responded to aa415-433 peptide, 8/23 to aa28-49 peptide, and 5 patients to both epitopes (Fig. 7D). Six of the eight patients showing a proliferative response to citrullinated aa415-433 peptide were analyzed for cytokine release (Supplementary Table S2). All patients released IFN γ , 3 of 6 released IL17, 2 of 6 TNF α , 2 of 6 IL4, 2 of 6 IL10, and 1 of 6 IL2.

Four of the six patients released at least three cytokines and 5 of 6 two cytokines. A normal donor also showed a strong proliferative response, which was CD4 mediated and specific to the citrullinated peptide (Fig. 7E). Analysis of the HLA restriction of responding patients showed that only one was HLA-DR4, suggesting these responses were not solely HLA-DR4 mediated and these peptides induce responses through a number of HLA-alleles.

Discussion

We provide the first evidence that citrullinated peptides can stimulate potent antitumor responses, suggesting that tumor cells present citrullinated peptides on MHC-II molecules. Furthermore, this presentation is autophagy and PAD dependent, although formal proof of the latter will require PAD knockout cell lines rather than just PAD inhibitors. Autophagy is triggered by stress such as hypoxia and nutrient starvation and is upregulated to promote tumor survival (21). PAD2 and PAD4 enzymes have also been shown to be expressed in a variety of cancer types and evidence of citrullinated proteins has been demonstrated in some cancer cell lines (22, 27, 28). We have shown that the efficient presentation of citrullinated peptides from vimentin onto MHC class II molecules is dependent upon autophagy, suggesting an important role for this process in presentation of modified peptide epitopes in tumor cells. The presentation of citrullinated peptides make excellent tumor targets, resulting in efficient CD4-mediated tumor clearance in melanoma and lung cancer models. The citrullinated vimentin-specific Th1 response was capable of tumor rejection even against established tumors and high tumor burden. Immunized mice demonstrating strong tumor rejection showed no evidence of toxicity, suggesting healthy cells do not present these modified epitopes. Indeed, it has been shown that RA cannot be induced by T cells alone but requires joint erosion, antibody responses and inflammation. This is borne out by studies where no autoimmune symptoms were observed with T cells alone, even in HLA-DR4 transgenic mice, which are susceptible to RA (29). However, the disease may be exacerbated in patients who already have RA and they will be excluded from any clinical studies. Potentially any cell undergoing autophagy could present citrullinated vimentin and be a target; however, we see no toxicity in the mouse models. We have shown that EBV transformed targets are recognized by splenocytes from immunized mice but this is perhaps not surprising as there is a degree of viral replication and cell lysis in these lines inducing some cellular stress. We speculate that any virally infected cell may also be a target but this should be beneficial rather than toxic. We do believe that chemotherapy may stress normal cells and the vaccine would need to be applied after recovery from the toxic effects of this therapy.

Frequent recognition of neo-epitopes by CD4 T cells in human melanoma has been observed (30). Indeed a recent analysis of responses to neo-epitopes within the B16 tumor showed the antitumor response to a CD4 neo-epitope was stronger than responses to CD8 neo-epitopes (31). In contrast with neo-epitopes, high-affinity T cells to self-antigens may be deleted. Recent studies using adoptively transferred TCR transgenic T cells highlight the important role of CD4 T cells in the direct targeting of tumors (32, 33). These studies relied on the use of TCR transgenic T cells as tolerance to the tumor antigen could not be overcome by vaccination. In contrast, we have observed robust CD4 responses

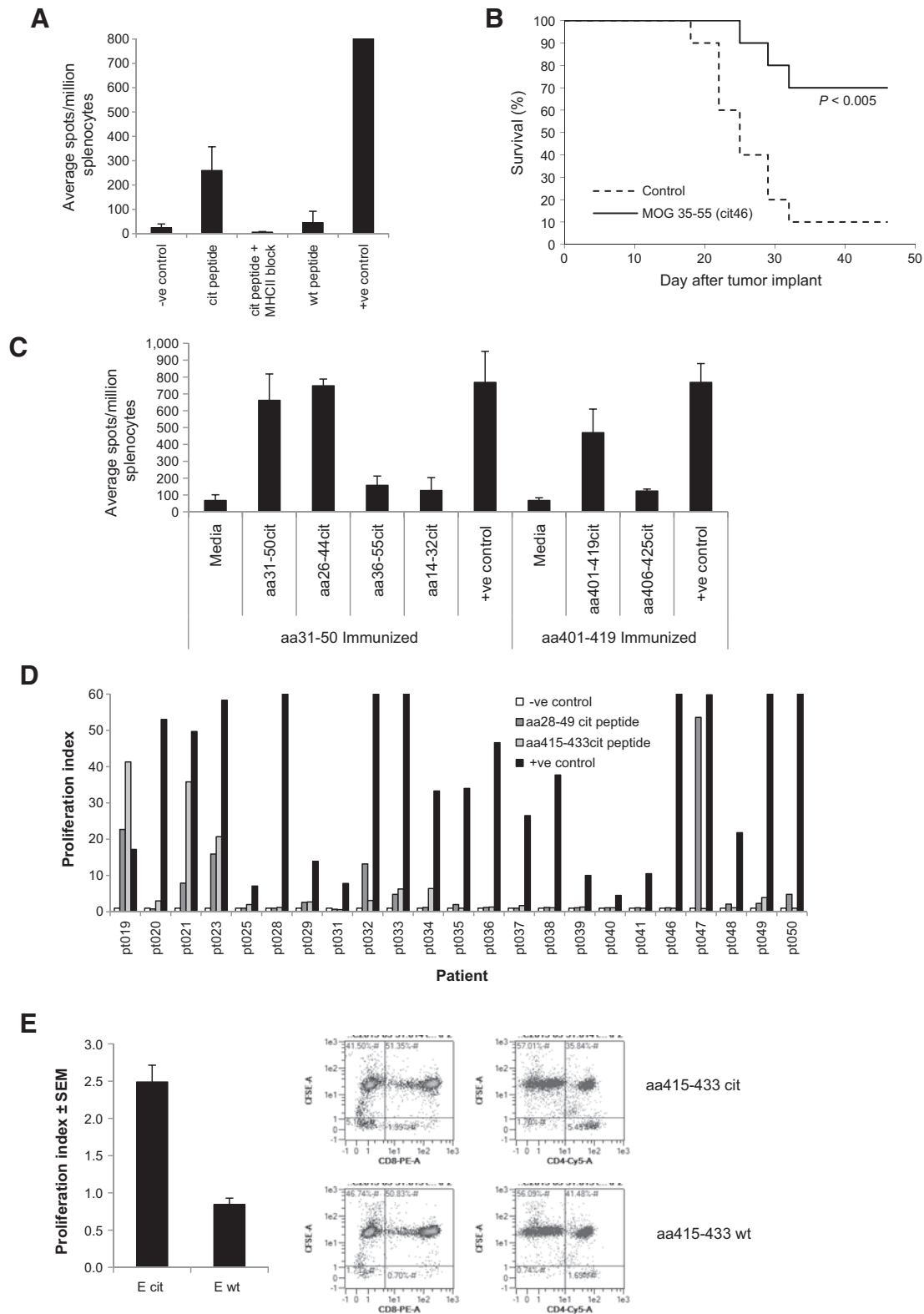


Figure 7. Are citrullinated proteins a novel class of tumor-associated antigen? A, C57Bl/6 mice were immunized with MOG 35-55 peptide citrullinated at position 46 and analyzed for IFN γ responses by Elispot assay. Representative of at least three independent experiments in which $n = 3$. Data are presented as mean \pm SD. B, survival of C57Bl/6 mice challenged with B16F1 tumor and vaccinated with MOG 35-55 (cit46) peptide at days 4, 7, and 11 ($n = 10$). C, C57Bl/6 mice were immunized with citrullinated aa31-50 or aa401-419 peptides and analyzed for IFN γ responses by Elispot assay. D, melanoma patient PBMCs were analyzed for proliferation to citrullinated aa28-49 or aa415-433 peptides by thymidine incorporation. E, normal donor PBMCs were analyzed for proliferation to aa415-433 citrullinated peptide by thymidine incorporation and CFSE dilution.

to vaccination with citrullinated peptides that resulted in antitumor immunity. Unlike neo-epitopes, this would not be patient specific and could be used to treat a wide range of cancers. The antitumor response induced by the citrullinated peptides was IFN γ and CD4 dependent, CD8 independent, and required direct recognition of HLA-DR4 on tumors. Although most tumors do not express MHC-II constitutively, they can be induced to express high levels by IFN γ . We have therefore produced a mouse tumor model that only upregulates HLA-DR4 in response to IFN γ . We see strong antitumor responses in this model. Our results compare favorably with responses to the Kif18b CD4 neo-epitope expressed within B16 tumors (31). Kreiter and colleagues showed 60% survival after eight immunizations with an RNA vaccine, commencing one day after tumor initiation. They show no antitumor response against established tumor when using a peptide vaccine encoding this epitope (34). We show 80% survival after a single immunization with a citrullinated peptide on day 10 after tumor initiation.

Of particular interest was the secretion of granzyme B in response to the citrullinated peptides and tumor cells expressing MHC class II. This recognition was inhibited by molecules that block both autophagy and citrullination. This suggested that the vaccination was inducing cytotoxic CD4 T cells. Cytotoxic CD4s have been difficult to induce by vaccination but adoptively transferred naïve CD4s recognizing self-antigens have been shown to differentiate to cytotoxic CTL (33) and this can be enhanced with OX40 engagement or following CD137/CD134 costimulation (35, 36). The citrullinated vimentin epitopes in this study induce secretion of IL17 and there is some evidence to suggest Th17 cells can also mediate tumor therapy; however, the effect mediated by these Th17 cells was also shown to be dependent upon IFN γ (37). Neutralization of IL17 in this study had minimal effects upon the antitumor response *in vivo* and there remains conflict on the relevance of Th17s in antitumor immunity (38). It is possible that these contradictory results could be due to the plasticity of Th17 cells *in vivo* (37, 39).

This study provides the first evidence that tumors can present citrullinated peptides as targets for CD4 cells. This presentation is dependent upon autophagy and PAD enzymes. The CD4 T cells

release IFN γ and show direct cytotoxicity, which results in potent antitumor responses *in vivo*. This approach is being fast tracked into the clinic in patient's whose tumors express vimentin. This could be patients with mesenchymal tumors or in patients whose tumors undergo epithelial to mesenchymal transition as vimentin is one of the first proteins to be expressed in this transition.

Disclosure of Potential Conflicts of Interest

V.A. Brentville has an ownership interest in a patent. R.L. Metheringham has ownership interest in a patent WO2014/023957. L.G. Durrant is the joint CEO of Scancell Ltd, receives commercial research grant from Scancell, has ownership interest (including patents) in Scancell Ltd, is a consultant/advisory board member of Scancell Ltd, and has provided expert testimony for Scancell Ltd. No potential conflicts of interest were disclosed by the other authors.

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